The maternal hormone in the male brain: sexually dimorphic distribution of prolactin signalling in the mouse brain

Sexual dimorphism in brain prolactin signalling

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ABSTRACT

Research of the central actions of prolactin is virtually restricted to females, but this hormone has also documented roles in male physiology and behaviour. Here, we provide the first description of the pattern of prolactin-derived signalling in the male mouse brain, employing the immunostaining of phosphorylated signal transducer and activator of transcription 5 (pSTAT5) after exogenous prolactin administration. Next, we explore possible sexually dimorphic differences by comparing pSTAT5 immunoreactivity in prolactin-supplemented males and females. We also assess the role of testosterone in the regulation of central prolactin signalling in males by comparing intact with castrated prolactin-supplemented males.

Prolactin-supplemented males displayed a widespread pattern of pSTAT5 immunoreactivity, restricted to brain centres showing expression of the prolactin receptor. Immunoreactivity for pSTAT5 was present in several nuclei of the preoptic, anterior and tuberal hypothalamus, as well as in the septofimbrial nucleus or posterodorsal medial amygdala of the telencephalon. Conversely, non-supplemented control males were virtually devoid of pSTAT5-immunoreactivity, suggesting that central prolactin actions in males are limited to situations concurrent with substantial hypophyseal

prolactin release (e.g. stress or mating). Furthermore, comparison of prolactin-supplemented males and females revealed a significant, female-biased sexual dimorphism, supporting the view that prolactin has a preeminent role in female physiology and behaviour. Finally, in males, castration significantly reduced pSTAT5 immunoreactivity in some structures, including the paraventricular and ventromedial hypothalamic nuclei and the septofimbrial region, thus indicating a region-specific regulatory role of testosterone over central prolactin signalling.

ABBREVIATIONS

3V	third ventricle
AAD	anterior amygdaloid area, dorsal part
AC/ADP	anterior commissural nucleus/anterodorsal preoptic nucleus region
ac	anterior commissure
Acb	nucleus accumbens
ACo	anterior cortical amygdaloid nucleus
AHi	amygdalohippocampal area
AHP	anterior hypothalamic area, posterior part
Aq	aqueduct (Sylvius)
Arc	arcuate hypothalamic nucleus
AVP	arginine-vasopressin
AVPe	anteroventral periventricular nucleus
Bar	Barrington´s nucleus
BLA	basolateral amygdaloid nucleus, anterior part
BLV	basolateral amygdaloid nucleus, ventral part
BMP	basomedial amygdaloid nucleus, posterior part
BSTIA	bed nucleus of the stria terminalis, intraamygdaloid division
BSTLD	bed nucleus of the stria terminalis, lateral division, dorsal part
BSTLP	bed nucleus of the stria terminalis, lateral division, posterior part
BSTLV	bed nucleus of the stria terminalis, lateral division, ventral part
BSTMA	bed nucleus of the stria terminalis, medial division, anterior part

BSTMV bed nucleus of the stria terminalis, medial division, ventral part

BSTMPI bed nucleus of the stria terminalis, medial division, posterointermediate part

BSTMPL bed nucleus of the stria terminalis, medial division, posterolateral part

BSTMPM bed nucleus of the stria terminalis, medial division, posteromedial part

cc corpus callosum

Ce central amygdala

CeC central amygdaloid nucleus, capsular part

CeL central amygdaloid nucleus, lateral division

CeM central amygdaloid nucleus, medial division

CPu caudate putamen (striatum)

CRF corticotropin-releasing factor

CxA cortex-amygdala transition zone

D3V dorsal third ventricle

DEn dorsal endopiriform nucleus

Dk nucleus of Darkschewitsch

DLG dorsal lateral geniculate nucleus

DLPAG dorsolateral periaqueductal grey

DM dorsomedial hypothalamic nucleus

DMPAG dorsomedial periaqueductal grey

DMTg dorsomedial tegmental area

DpG deep grey layer of the superior colliculus

DpMe deep mesencephalic nucleus

DpWh deep white layer of the superior colliculus

DR dorsal raphe nucleus

DTg dorsal tegmental nucleus

f fornix

fi fimbria of the hippocampus

HDB nucleus of the horizontal limb of the diagonal band

I intercalated nuclei of the amygdala

85	ic	internal capsule
86	IF	interfascicular nucleus
87	InC	interstitial nucleus of Cajal
88	InG	intermediate grey layer of the superior colliculus
89	InWh	intermediate white layer of the superior colliculus
90	IPAC	interstitial nucleus of the posterior limb of the anterior commissure
91	La	lateral amygdaloid nucleus
92	LA	lateroanterior hypothalamic nucleus
93	LC	locus coeruleus
94	LDTg	laterodorsal tegmental nucleus
95	LGP	lateral globus pallidus
96	LH	lateral hypothalamic area
97	LHb	lateral habenular nucleus
98	lo	lateral olfactory tract
99	LOT	nucleus of the lateral olfactory tract
100	LPAG	lateral periaqueductal grey
101	LPBC	lateral parabrachial nucleus, central part
102	LPBD	lateral parabrachial nucleus, dorsal part
103	LPBE	lateral parabrachial nucleus, external part
104	LPBI	lateral parabrachial nucleus, internal part
105	LPBV	lateral parabrachial nucleus, ventral part
106	LSD	lateral septal nucleus, dorsal part
107	LSI	lateral septal nucleus, intermediate part
108	LSV	lateral septal nucleus, ventral part
109	LV	lateral ventricle
110	MCLH	magnocellular nucleus of the lateral hypothalamus
111	mcp	middle cerebellar peduncle
112	MCPO	magnocellular preoptic nucleus
113	ME	median eminence

114	MeA	medial amygdaloid nucleus, anterior part
115	MePD	medial amygdaloid nucleus, posterodorsal part
116	MePV	medial amygdaloid nucleus, posteroventral part
117	MGD	medial geniculate nucleus, dorsal part
118	MGM	medial geniculate nucleus, medial part
119	MGP	medial globus pallidus
120	MGV	medial geniculate nucleus, ventral part
121	MHb	medial habenular nucleus
122	ml	medial lemniscus
123	mlf	medial longitudinal fasciculus
124	MnPO	median preoptic nucleus
125	MnR	median raphe nucleus
126	MPA	medial preoptic area
127	MPB	medial parabrachial nucleus
128	MPBE	medial parabrachial nucleus, external part
129	MPO	medial preoptic nucleus
130	MS	medial septal nucleus
131	mt	mammilothalamic tract
132	MTu	medial tuberal nucleus
133	MZMGV	marginal zone of the medial geniculate
134	opt	optic tract
135	OT	oxytocin
136	ox	optic chiasm
137	Pa	paraventricular nucleus
138	PaLM	paraventricular nucleus, lateral magnocellular part
139	PaMM	paraventricular nucleus, medial magnocellular part
140	PaPO	paraventricular nucleus, posterior part
141	PaV	paraventricular nucleus, ventral part
142	PAG	periaqueductal greyPe periventricular hypothalamic nucleus

143	PeF	perifornical nucleus
144	PH	posterior hypothalamic area
145	PIL	posterior intralaminar thalamic nucleus
146	Pir	piriform cortex
147	PLCo	posterolateral cortical amygdaloid nucleus
148	PnC	pontine reticular nucleus, caudal part
149	PnO	pontine reticular nucleus, oral part
150	PnR	pontine raphe nucleus
151	PnV	pontine reticular nucleus, ventral part
152	PoT	posterior thalamic nuclear group, triangular part
153	PP	peripeduncular nucleus
154	PRL	prolactin
155	PSTh	parasubthalamic nucleus
156	PV	paraventricular thalamic nucleus
157	PVA	paraventricular thalamic nucleus, anterior part
158	RCh	retrochiasmatic area
159	RMg	raphe magnus nucleus
160	s5	sensory root of the trigeminal nerve
161	SCh	suprachiasmatic nucleus
162	SFi	septofimbrial nucleus
163	SFO	subfornical organ
164	SG	supragenicular thalamic nucleus
165	SI	substantia innominata
166	SLEA	sublenticular extended amygdala
167	sm	stria medullaris of the thalamus
168	SNC	substantia nigra, pars compacta
169	SNR	substantia nigra, reticular part
170	SO	supraoptic nucleus
171	SPa	subparaventricular zone of the hypothalamus

st	stria terminalis
STh	subthalamic nucleus
SubCD	subcoeruleus nucleus, dorsal part
SubCV	subcoeruleus nucleus, ventral part
SuM	supramammillary nucleus
Te	terete hypothalamic nucleus
Tu	olfactory tubercle
VEn	ventral endopiriform nucleus
VLG	ventral lateral geniculate nucleus
VLGMC	ventral lateral geniculate nucleus, magnocellular part
VLGPC	ventral lateral geniculate nucleus, parvicellular part
VLPAG	ventrolateral periaqueductal grey
VLPO	ventrolateral preoptic nucleus
VMH	ventromedial hypothalamic nucleus
VMHc	ventromedial hypothalamic nucleus, central part
VMHdm	ventromedial hypothalamic nucleus, dorsomedial part
VMHvl	ventromedial hypothalamic nucleus, ventrolateral part
VMPO	ventromedial preoptic nucleus
VOLT	vascular organ of the lamina terminalis
VP	ventral pallidum
VRe	ventral reuniens thalamic nucleus
vsc	ventral spinocerebellar tract
VTA	ventral tegmental area
ZI	zona incerta

197 **INTRODUCTION**

198 Prolactin (PRL) is a polypeptide hormone produced at the adenohypophysis, best-known for its role
199 in the development of the mammary gland and milk production (1). To date, PRL has been attributed
200 more than 300 different biological actions (2) in reproduction, homeostasis, angiogenesis or
201 immunity, among others (3). To fulfil this array of functions, PRL has different target tissues,
202 including the mammary gland, the uterus and other peripheral organs (3) and, importantly, the brain
203 (4,5). In the brain, PRL is mainly involved in the regulation of a number of behaviours and
204 physiological processes related to female reproduction and lactation (6) . Indeed, PRL has been
205 described as the "maternal hormone", a pleiotropic hormone responsible for adapting every
206 important aspect of female physiology and behaviour to the demands of motherhood (7).

207 The key role of prolactin in maternal physiology has traditionally biased neuroendocrinological
208 studies towards its functions in the brain of females, whereas the actions of this hormone in the
209 male brain have drawn less attention. For instance, the distribution of the PRL receptor (PRLR) has
210 been reported for the female (8,9) but not the male rodent brain. One of the main reasons for this
211 bias relates to the fact that male rodent models display low levels of circulating PRL under standard
212 conditions (10), reflecting a limited access and functionality of PRL in the brain. Still, several studies
213 do report acute rises in systemic PRL secretion in males associated to certain physiological
214 conditions, such as the stress response (11) or sexual behaviour (12).

215 Regarding the latter, PRL is known to be involved in the control of male copulatory behaviour in rats
216 (13), mice (14), other rodent models (15) and humans (16). Thus, PRL is released acutely with
217 ejaculation (12) and it is proposed to intervene in the satiation following copulation(17) leading to
218 the refractory period. In fact, chronic hyperprolactinaemia suppresses copulatory behaviour in
219 animal models(18). In men, hyperprolactinaemia associated to several clinical conditions (19)
220 (including antipsychotic treatments (20)), yields sexual dysfunction and other side-effects (21). The
221 inhibitory role of PRL on sexual function is proposed to have an important central component

222 (22,23), but the exact regions and mechanisms through which PRL operates in the male brain are still
223 poorly understood.

224 In this light, the main aim of this work is to provide a comprehensive description of the distribution
225 of cells sensitive to PRL in the brain of males. To do so, we have supplemented males with a
226 standard dose of exogenous PRL and revealed PRL-responsive cells in the brain by means of the
227 immunohistochemical detection of the phosphorylated form of signal transducer and activator of
228 transcription 5 (pSTAT5). Phosphorylation of STAT5 is a key event in the Jak/STAT pathway, the
229 major signalling cascade of the PRL receptor (3). In contrast to other methods, e.g. in situ
230 hybridization of the PRL receptor (PRLR) mRNA (or its immunodetection), pSTAT5
231 immunohistochemistry in PRL-supplemented animals reveals those cells that are not just expressing
232 PRL receptors or specific elements of their signalling cascade, but those that are fully responsive to
233 PRL. Thus, pSTAT5 immunodetection can be considered a reliable method to assess central PRL
234 responsiveness (4,5)

235 The second goal of this work is to compare the pattern of central PRL-derived signalling between
236 male and female mice, in search for intersexual differences. For this purpose, we compare
237 qualitatively and quantitatively the patterns of our PRL-supplemented males with ovariectomised,
238 steroid-primed females treated with an equivalent dose of PRL. In addition, a direct comparison of
239 the distribution of pSTAT5 in the brain of males and females (where most functional studies on the
240 matter have been conducted) might also be useful for the functional contextualization of PRL input
241 to the male brain.

242 In accordance with the involvement of PRL in reproductive physiology and behaviour, gonadal
243 steroids are important regulators of PRL function in the brain. In female rodents, estradiol and
244 progesterone have been shown to target most PRL-sensitive neurons in the brain (24) and to
245 regulate PRL signalling at different levels (25–27). On the other hand, testosterone has been
246 documented to inhibit PRL receptor expression in the pituitary gland of male rats and pituitary PRL

247 release in male rats and mice (28–30). Beyond these findings, to our knowledge there is currently no
248 available evidence supporting a regulatory role of testosterone in brain PRL signalling. Hence, in this
249 work we also explore the putative contribution of testosterone in shaping the patterns of PRL
250 responsiveness in the male brain. To this end, we analyse the levels of pSTAT5-ir in the main PRL-
251 responsive regions of the male mouse brain after permanent testosterone withdrawal through
252 castration, as compared to intact male mice, both supplemented with PRL.

253 By characterizing the patterns of PRL signalling in the male brain, assessing its sexual dimorphism
254 and clarifying their dependence on testosterone, we intend to shed light on the roles of PRL in male
255 behaviour and physiology, as well as on the neural substrates for PRL-dependent, male-specific
256 behaviours.

257 **MATERIAL AND METHODS**

258 **Animals**

259 For the present study, we used 15 male and 6 female mice of the CD1 strain (Charles River
260 Laboratories, France), aging between 8 and 24 weeks. These animals were housed in polypropylene
261 plastic cages under controlled temperature (24 ± 2 ºC) and lighting conditions (12h:12h; lights ON at
262 8 am), with *ad libitum* access to food and water. Intact males were housed individually (to avoid
263 inter-male aggression) whereas castrated males and females were group-housed (4 to 6 animals per
264 group, to avoid isolation-derived stress). Animals were treated throughout according to the
265 European Union Council Directive 2010/63/EU (6106/1/10 REV1) and procedures were approved by
266 the Committee of Ethics on Animal Experimentation of the University of Valencia (protocol number:
267 2015/VSC/PEA/00055), where the experiments were performed.

268 **Experimental Design**

269 Male mice were randomly assigned to three experimental groups: (1) Male Control (n=3); (2)
270 Male+PRL (n=6) and (3) Castrated+PRL (n=6). Animals of Male Control and Male+PRL groups were

271 left gonadally intact, whereas males from Castrated+PRL group underwent orchidectomy. Following
272 the procedure by Brown and collaborators (5,31), Male+PRL and Castrated+PRL groups received an
273 exogenous PRL supplementation 45 minutes prior to perfusion.

274 In order to compare central PRL sensitivity between males and females, we also included in our
275 study a group of ovariectomised, steroid-primed and PRL-supplemented females, which belonged to
276 a sample of a previously published set of experiments (4). The steroid-replacement schedule
277 conducted on these females intended to emulate the proestrus-estrus phase of the estrous cycle
278 (32). Given the considerable variability of pSTAT5-ir in the brain of freely-cycling females, likely due
279 to gonadal steroid influence on PRL signalling (4), this group provided a more stable comparison
280 than freely-cycling females and a more accurate comparison than estrous cycle screening.

281 Data from these four groups of animals (Male Control; Male+PRL; Castrated+PRL and Female+PRL)
282 allowed us to: a) characterize the pattern of PRL-derived signalling in the male brain by analysing the
283 distribution of pSTAT5-ir in group Male+PRL; b) analyse the effect of PRL supplementation on
284 pSTAT5-ir distribution by comparing the Male Control with Male+PRL groups; c) explore sexual
285 dimorphism in the central sensitivity for PRL by comparing Male+PRL with Female+PRL groups; and
286 finally, d) assess the modulatory role of testosterone on PRL signalling in the male brain through the
287 comparison of the Male+PRL and the Castrated+PRL groups.

288 **Ovariectomy and Orchidectomy**

289 Males of Castrated+PRL group were orchidectomised at approximately 12 weeks of age. Briefly,
290 animals were deeply anaesthesised with i.p. ketamine (Imalgene 500, Merial, Toulouse, France,
291 75mg/kg) and medetomidine (Domtor 1mg/ml, Esteve, Barcelona, Spain, 1 mg/kg) and surgery was
292 performed via a single midline incision on the scrotal sac. After surgery, i.p. atipamezol
293 hydrochloride (Antisedan, Pfizer, New York, USA, 1 mg/kg) was administered to reverse anaesthesia
294 and s.c. butorphanol tartrate 1% (Torbugesic, Pfizer, New York, USA, 20 µl) was regularly

295 administered through the postsurgical period for pain control. Castrated males had 2-3 weeks of
296 recovery under undisturbed housing conditions before the beginning of the experiments.

297 Experimental females underwent ovariectomy 9-10 weeks after birth under identical
298 pharmacological conditions as explained for males (4). Surgery was conducted through two incisions
299 on both sides of the back of the animal. Ovariectomized females had at least seven days of recovery
300 after surgery.

301 **Hormone Treatments**

302 Experimental females underwent a steroid replacement schedule (4) consisting of a sustained
303 treatment with 17-β estradiol and an acute treatment with progesterone. In accordance with the
304 experimental induction of estrus (32), estradiol was administered on a slow-release profile during 7
305 days, by means of the subcutaneous placement of silastic tubing implants (Dow Corning Corporation,
306 Midland, MI, USA) filled with 20 µg/ml β-estradiol (Sigma, St Louis, MO, USA) diluted in sunflower
307 oil. Silastic tubing had an inner diameter of 1.67 mm and an outer diameter of 2.41 mm, and
308 implants were cut to a length of 20 mm. Implants were inserted subcutaneously on the lumbar
309 region under isoflurane anaesthesia (Isoflo, Esteve Veterinaria, Barcelona, Spain). Animals were also
310 administered a subcutaneous dose of 20 µl butorphanol tartrate 1% (Torbugesic, Pfizer, New York)
311 during implant placement surgery, for pain control. By contrast, progesterone (Sigma, St Louis, MO,
312 USA) was administered to females acutely in a 500 µg subcutaneous injection, diluted in sunflower
313 oil, in the morning of the seventh day of estradiol treatment, specifically 3 hours and 45 minutes
314 prior to perfusion. In order to make males and females directly comparable for the analysis of sexual
315 dimorphism, males of the Male+PRL group received vehicle treatment parallel to that of females
316 (implants with sunflower oil, plus s.c. oil injection 3h 45 minutes prior to perfusion).

317 Regarding PRL supplementation, except for the Male Control group (which received vehicle
318 injection), all experimental groups were administered an acute 5 mg/kg i.p. dose of ovine PRL
319 (Sigma, St Louis, MO, USA) 45 minutes before perfusion. The dose and timing of PRL administration

320 rendered homogenous, supraphysiological circulating levels of PRL and ensured that the peak of
321 STAT5 phosphorylation in response to a PRL challenge coincided with perfusion (5). This is intended
322 to reveal the maximal potential response patterns among experimental animals and also to allow
323 direct comparison between experimental groups, regardless of sex (male vs female) or physiological
324 condition (intact vs castrated males).

325 **Tissue Collection and Histological Processing**

326 Animals received an overdose of sodium pentobarbital (Vetoquinol, Madrid, Spain) and were
327 transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Brains
328 were carefully extracted and post-fixed overnight through immersion in the same fixative, then
329 cryoprotected by immersion in 30% sucrose in 0.01 M PB until they sank (2-3 days). Using a freezing
330 microtome (Microm HM-450, Walldorf, Germany) brains were sectioned in five parallel series of 40
331 µm thick coronal sections. Series were stored in PB-30% sucrose at -20ºC until their use.

332 **Immunohistochemistry for pSTAT5**

333 Immunohistochemistry was conducted in free-floating sections under light shaking at room
334 temperature (25ºC) unless otherwise stated. Immunohistochemistry protocol was adapted from
335 Brown et al.(5,31). Tissue sections were thoroughly rinsed between stages for at least three 10-min
336 washes in TRIS-buffered saline, 0.05M, pH 7.6 (TBS). After thawing, sections underwent an initial
337 antigen retrieval step, consisting in two sequential 6-minute incubations in 0.01 M TRIS buffer (TB),
338 pH 10 at 85ºC, and brought quickly to room temperature in between. Tissue was then incubated in:
339 a) 1% hydrogen peroxide (H_2O_2) for 30 minutes, for endogenous peroxidase inhibition; b) 2% BSA,
340 2% goat serum and 0.3% Triton X-100 in TBS for 1h, in order to block unspecific labelling; c) rabbit
341 monoclonal anti-pSTAT5 primary antibody (Phospho-Stat5 Tyr694 (D47E7), Catalog number #4322,
342 Cell Signaling Technology, Danvers, MA) diluted 1:500 in TBS plus Triton X-100 0.1% for 72 h at 4ºC;
343 d) biotinylated goat anti-rabbit IgG (Vector Laboratories, Peterborough, UK; RRID AB_2313606)
344 1:200 in TBS for 90 minutes; and e) avidin-biotin-peroxidase complex (ABC Elite kit; Vector

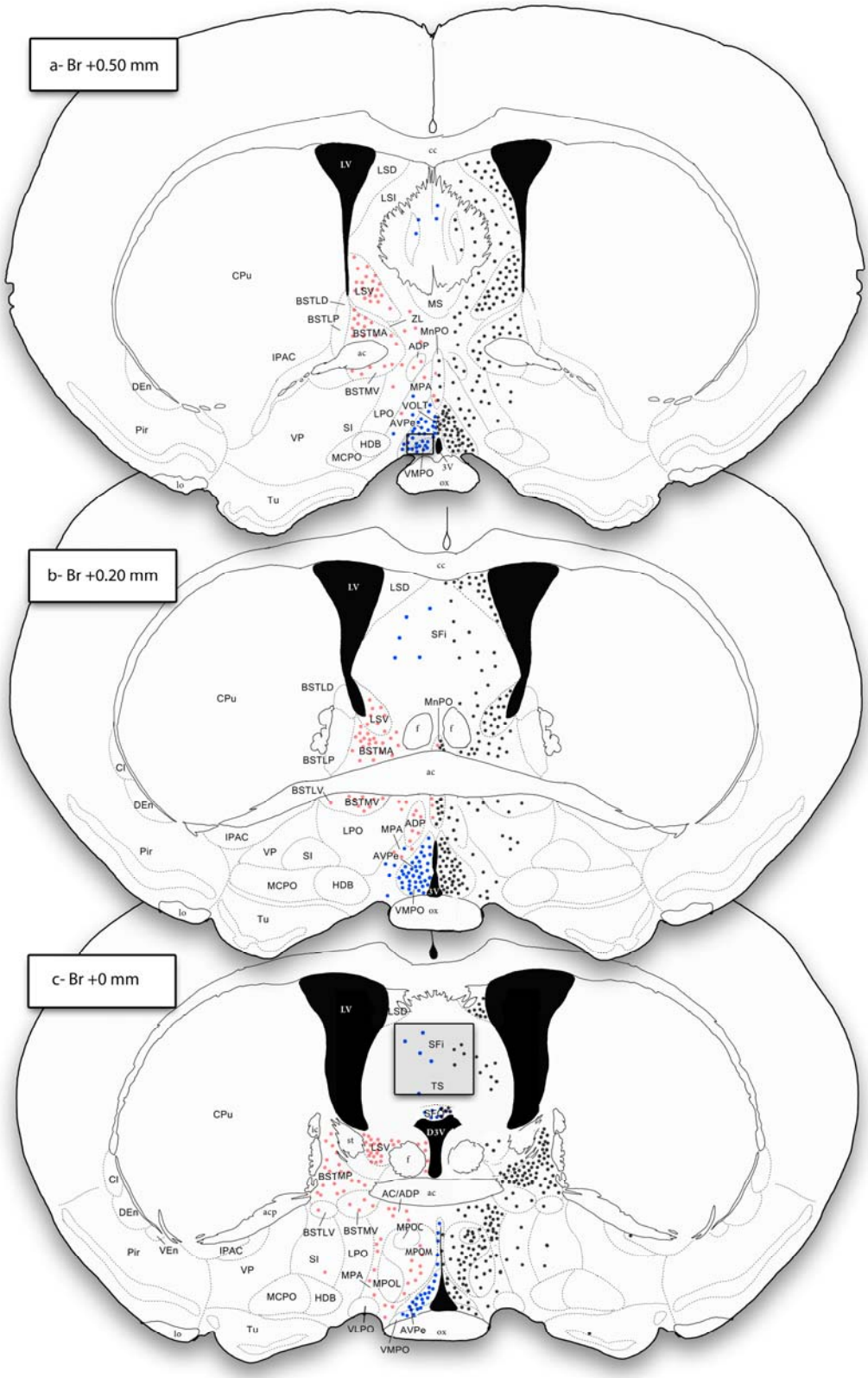
345 Laboratories; RRID AB_2336819) in TBS for 90 minutes. Peroxidase label was developed using
346 0.005% 3-3'-diaminobenzidine (Sigma) and 0.01% H_2O_2 in TB pH 7.6 for about 15 minutes, obtaining
347 thereby a brown nuclear staining. Sections were rinsed in TB and mounted onto gelatinized slides,
348 dehydrated in graded ethanol, cleared with xylene and coverslipped with Entellan.

349 The specificity of the anti-pSTAT5 primary antibody was demonstrated by means of an antibody
350 competition assay (Fig S1).

351 **Analysis of pSTAT5 Immunoreactivity**

352 The patterns of pSTAT5-ir distribution were mapped for the brain of intact males (Male+PRL) and
353 females (Female+PRL) and illustrated semiquantitatively in camera lucida drawings of selected brain
354 sections (Fig 1). In addition, a quantitative assessment of the density of cells showing pSTAT5
355 immunoreactivity (pSTAT5-ir) in representative brain sites was performed for PRL-treated females
356 and males, both intact and castrated. To do so, we first selected frames of the chosen nuclei using
357 the stereotaxic atlas of Paxinos and Franklin (2004). Then, we obtained photomicrographs of these
358 frames in both hemispheres using a digital camera (Leica DFC495) attached to a microscope Leitz
359 DMRB (Leica AG, Germany). Thereafter, images were processed and analysed using ImageJ. Briefly,
360 we subtracted background light and converted the RGB colour image to greyscale by selecting the
361 green channel. Then, we binarised the greyscale image setting the 75% of the mode of the histogram
362 as a threshold, thus including every pixel below this threshold as positively labelled. Using the
363 ImageJ commands "fill holes", "open" (3 iterations) and "watershed", we filtered small particles
364 (noise or fragmented nuclei) and separated fused objects. The resulting particles were additionally
365 filtered by area (larger than 70 μm^2, corresponding to an approximate diameter of 9.4 µm) and
366 finally counted automatically. We calculated the mean (interhemispheric) density of pSTAT5-
367 imunoreactive cell nuclei for each specimen by dividing the counts for both hemispheres by the area
368 of both frames.

369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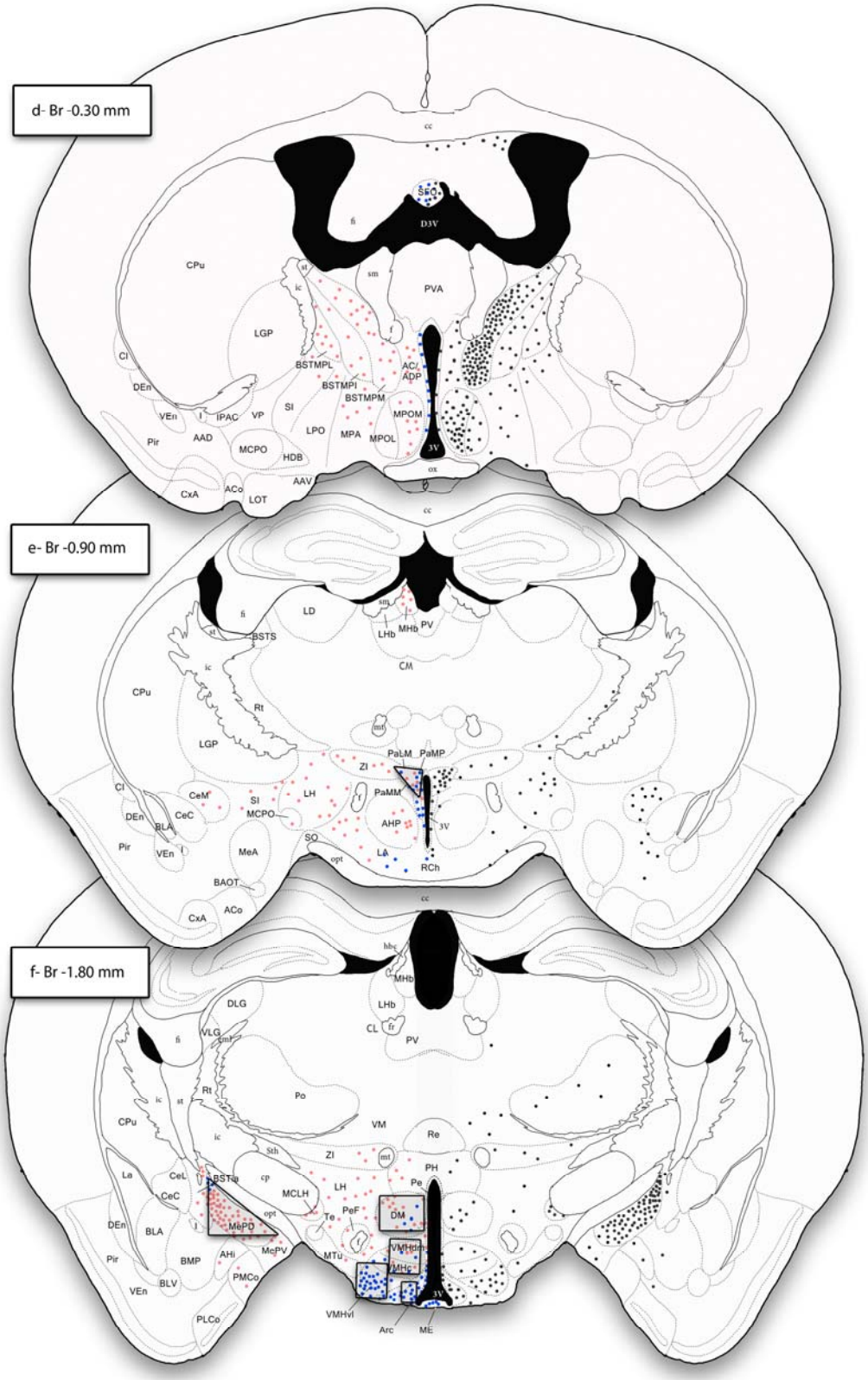
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372

373 **Figure 1. Mapping of pSTAT5 immunoreactivity and prolactin receptor expression in the brains of male and**
374 **female mice**

375 Semi-schematic camera lucida drawings of coronal sections of the mouse brain showing the distribution patterns of
376 pSTAT5-ir in female and male mice (left side) and the pattern of PRLR expression in the brain of an adult male mouse
377 specimen (right side), as determined by PRLR expression data obtained from the Allen Brain Institute (© 2004 Allen
378 Institute for Brain Science. Allen Mouse Brain Atlas. Available from: mouse.brain-map.org experiment 72340223,
379 http://mouse.brain-map.org/experiment/show/72340223). In the left side, pink dots represent pSTAT5 expression
380 exclusive of ovariectomized, steroid-primed females, whereas dark blue dots encode overlapping expression of
381 pSTAT5-ir in both the female and male specimens. Black dots in the right illustrate PRLR expression in the male
382 mouse brain. Each dot represents approximately 4 cell nuclei labelled for pSTAT5 or equivalent number of cells
383 expressing the PRLR. Shaded areas indicate the counting frames designed for the AVPe/VMPO region (Fig 1a), the
384 Pa (Fig 1e), and the Arc, VMHvl, VMHc and VMHdm, DM and MePD (all frames in Fig 1f), as part of the quantitative
385 analysis performed in this work (see below). Approximate distance to bregma is indicated for each section.

386

387 **Statistical Analysis**

388 We performed statistical analysis of the resulting data on the SPSS software package. After checking
389 for normality (Kolmogorov-Smirnov test with Lilliefors' correction) and homogeneity of variances
390 (Levene's test), we conducted three different statistical comparisons. First, we compared levels of
391 pSTAT5-ir density between intact males (Males + PRL) and ovariectomised, steroid-primed females
392 (Female+PRL), in search for dimorphic differences. Then, we checked for the effect of orchidectomy
393 on male pSTAT5-ir levels by comparing intact (Male+PRL) and castrated (Castrated+PRL) males. For
394 both analyses, the samples fulfilling the criteria for a parametric analysis were subject to an
395 independent t-test, whereas the samples of the remaining nuclei were subject to a non-parametric
396 Mann-Whitney test. For each statistical test, we applied a significance level of 0.05.

397 **RESULTS**

398 The main focus of this section is to characterise the central patterns of pSTAT5-ir found in male mice
399 in the presence of high levels of circulating PRL (Male+PRL group), as an indicator of PRL-derived
400 signalling occurring in the male brain. Likewise, we compare the distribution of pSTAT5-ir between
401 PRL-supplemented males (Male+PRL group) and non-supplemented males (Male Control group).
402 Then, we analyse intersexual differences in PRL-derived signalling in the mouse brain. For this
403 purpose, we compare the patterns of pSTAT5-ir in the male (Male+PRL group) and female
404 (Female+PRL group) brains and assess the density of pSTAT5-ir in a number of brain regions
405 displaying PRL-derived signalling in both males and females. Finally, we explore the effect of
406 testosterone withdrawal in the PRL-derived signalling of the male brain by comparing pSTAT5-ir
407 levels between intact and castrated male mice. For the following anatomical descriptions, we adhere
408 to the neuroanatomical terminology proposed by Paxinos and Franklin(33) (see also list of
409 abbreviations above).

410 **A. Patterns of pSTAT5 immunoreactivity in the male mouse brain: effect of PRL supplementation**

411 Immunohistochemistry for pSTAT5 produced a defined staining in the examined brain tissue, which
412 was restricted to the cell nucleus (see Fig 2), as shown in our previous work (4). Labelling was
413 suppressed by pre-incubation of the antibody with the phosphorylated peptide used for
414 immunization, but not when the antibody was incubated with the equivalent, non-phosphorylated
415 peptide (Fig S1). Therefore, we safely assume that, in our immunohistochemical procedure, the
416 antibody is detecting specifically pSTAT5.

417

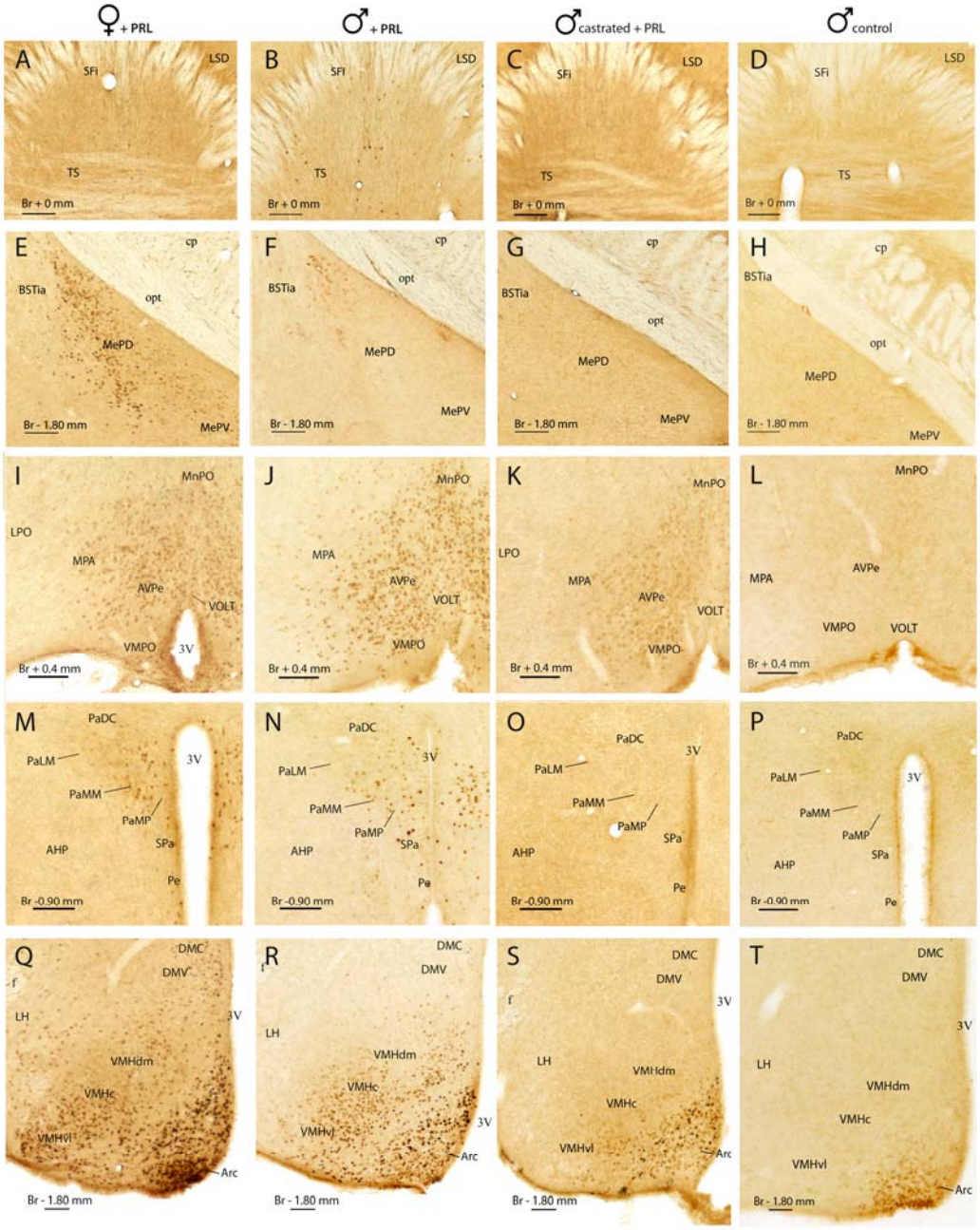


Figure 2. Representative examples of pSTAT5 immunoreactivity in the brain of females, intact males and castrated male mice

Photomicrographs illustrating pSTAT5 labelling in representative brain sections in an ovariectomized, steroid-primed female supplemented with PRL (I), an intact, PRL-supplemented male (II), a castrated, PRL supplemented male (III) and a intact control male lacking PRL supplementation (IV). Sections correspond to the preoptic hypothalamus (A), the paraventricular region in the anterior hypothalamus (B), the tuberal hypothalamus (C), the caudal septum (D) and

425 the medial posterior amygdala (E). The approximate distance to bregma is indicated in each section. Scale bars
426 correspond to 100 μm.

427 Figure 1 shows the distribution of pSTAT5-ir observed in PRL-supplemented males (blue dots on the
428 left side of the brain), as well as the the distribution of cells expressing PRL receptor (PRLR) in the
429 male mouse brain (Fig 1, black dots on the right side of the brain), as reported by the Allen Brain
430 Institute (Allen Mouse Brain Atlas, experiments 1268 and 72340223, Fig S2). Figure 2 shows
431 photomicrographs illustrating pSTAT5-ir in selected brain nuclei for all of our experimental groups.

432 Males supplemented with PRL (Male+PRL) showed moderate-to-dense pSTAT5-ir in several nuclei of
433 the hypothalamus and cerebral hemispheres (Fig 2, left column), as well as in the choroid plexus (not
434 shown). In the telencephalon, pSTAT5-ir was mainly observed in the septum, specifically in the area
435 comprising the septofimbrial nucleus (SFi) and the triangular septal nucleus (TS) (Figs 1B and 1C). In
436 this region, the subfornical organ (SFO) also showed some immunoreactive cells. In addition, the
437 posterodorsal nucleus of the medial amygdala (MePD) displayed scarce pSTAT5-ir, with few
438 immunolabelled cells restricted to the upper corner of the nucleus, in close contact to the
439 intramygdaloid BST (BSTia, see Figs 1F and 2F). Importantly, all these brain nuclei display PRLR mRNA
440 expression in the male mouse (Fig 2 right side).

441 In the hypothalamus, pSTAT5-ir was present in the preoptic, anterior and tuberal regions. In the
442 preoptic hypothalamus (Figs 1A-D and 2J), pSTAT5-ir cells were concentrated in the juxtaventricular
443 nuclei, namely the vascular organ of the *lamina terminalis* (VOLT) and the anteroventral
444 periventricular (AVPe), the ventromedial preoptic (VMPO) and the Pe nuclei. In addition, the
445 adjoining MPA displayed a few immunolabelled cell nuclei.

446 In the anterior hypothalamus, pSTAT5-ir was once again restricted to juxtaventricular structures, the
447 paraventricular nucleus (Pa) and the Pe (Figs 1E and 2N), as well as the retrochiasmatic area (RCh,
448 Fig 1E). In the Pa, labelling was restricted to the medial aspect of the nucleus, mainly to the ventral
449 (PaV), medial magnocellular (PaMM) and medial parvocellular (PaMP) subdivisions, with very few

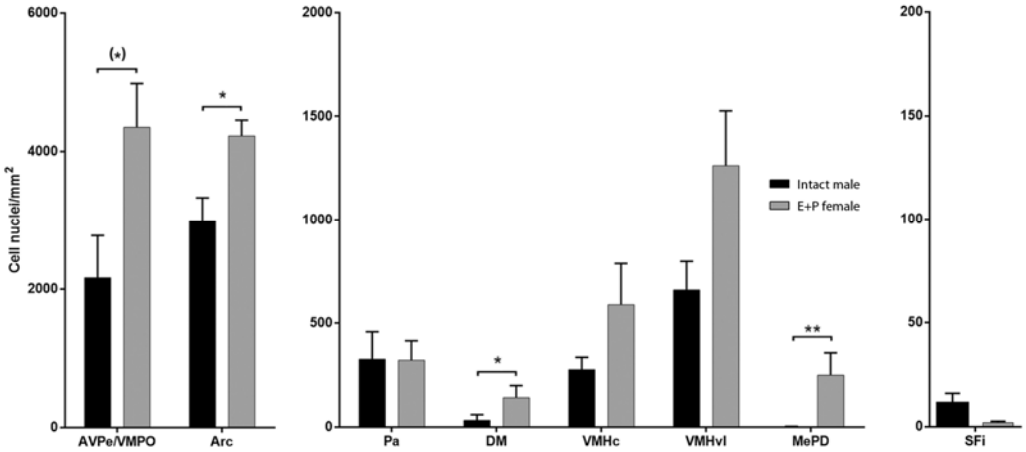
450 labelling observed in the lateral aspect of the nucleus (PaLM) or the subparaventricular nucleus
451 (SPa). In the tuberal hypothalamus (Figs 1F and 2R), the arcuate nucleus (Arc) displayed abundant
452 pSTAT5-ir, with some immunolabelled cells displaced into the median eminence (ME). In some
453 males, the dorsomedial nucleus (DM) displayed few scattered immunolabelled cells. Finally, pSTAT5-
454 ir appeared in the ventromedial hypothalamic nucleus (VMH), with higher levels in the ventrolateral
455 subdivision (VMHvl) and sparse labelling in the rest (VMHc and VMHdm).

456 In contrast to the Male+PRL group, non-supplemented males (Male Control, Fig 2, rightmost
457 column), were virtually devoid of pSTAT5-ir, except for some labelled cells in the ventral aspect of
458 the arcuate nucleus (Arc, Fig 2T) and the anterior aspect of the periventricular nucleus (Pe, not
459 shown).

460 **B. Comparative analysis of pSTAT5-ir the male and female mouse brain**

461 In comparison to males, ovariectomised, steroid-primed females supplemented with PRL
462 (Female+PRL) showed a more extensive distribution of pSTAT5-ir (Fig 1, Fig 2 middle-right column),
463 which matches previous descriptions conducted on freely-cycling female mice (4). To summarize,
464 labelling in our experimental females was widespread in the basal telencephalon and hypothalamus,
465 but was also found within thalamic, midbrain and brainstem structures. To allow a direct comparison
466 between sexes, Figure 2 shows the distribution of pSTAT5-ir in equivalent brain sections of male and
467 female mice. In addition, in the semischematic drawings of brain sections depicted in Fig 1, pSTAT5-
468 ir labelling that was common to males and females is shown as blue spots, whereas labelling
469 exclusively present in females is illustrated as red spots. Importantly, there was not a single brain
470 site where labelling was exclusively found in males. In other words, after administration of
471 equivalent doses of exogenous PRL, males show pSTAT5-ir in a reduced number of brain centres,
472 whereas females show immunoreactive cells in the same nuclei, plus a set of additional brain
473 centres. This represents a clear case of sexual dimorphism in favour of females.

474 We also checked for quantitative sexual dimorphism in PRL-derived signalling, by analysing the
475 density of pSTAT5-ir in the main nuclei showing pSTAT5-ir in PRL-supplemented intact males and
476 steroid-primed ovariectomised females. We designed counting frames for the following brain nuclei:
477 the AVPe/VMPO region, the Pa, the Arc, the DM, the VMHvl, the area comprising the central and
478 dorsomedial VMH (VMHc and VMHdm, respectively), the MePD and the SFi (Fig 1). These results are
479 summarized in Figure 3. Females showed a general trend towards higher levels of pSTAT5-ir than
480 males. This effect reached statistical significance in the Arc (t(10)=3.043, p=0.012), the DM (U=32.0;
481 p=0.026) and the MePD (U=30.0; p=0.004), although the AVPe/VMPO also showed an almost
482 significant trend towards higher pSTAT5-ir density in females (t(9)=2.183; p=0.057).

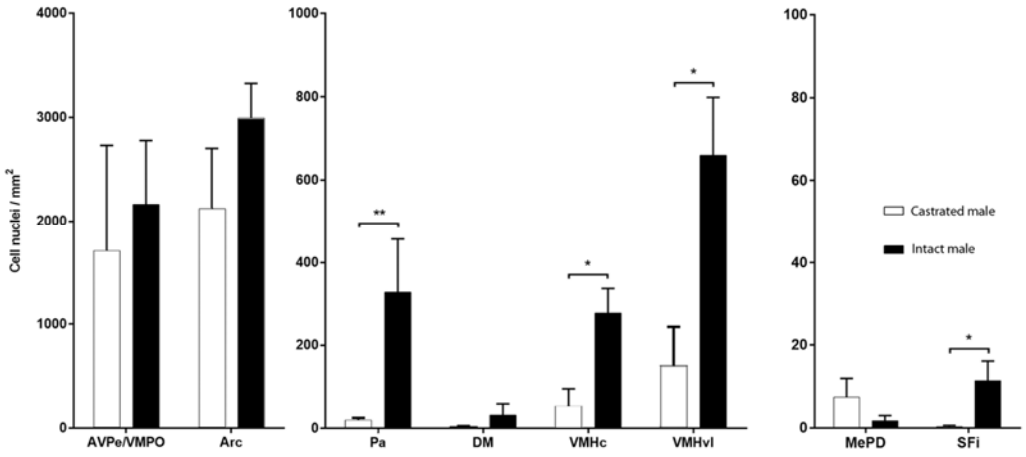
483 

484 **Figure 3. Quantitative analysis of pSTAT5 immunoreactivity in selected brain regions of female and male mice**

485 Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei/mm^2) in the major brain regions with expression of
486 pSTAT5-ir in both male and female mice. Bar histograms show mean interhemispheric pSTAT5-ir density ± SEM in
487 gonadally-intact, vehicle-treated males (Male+PRL; n=6; black) and ovariectomized females treated with estradiol and
488 progesterone (Female+PRL; n=6; grey). Counting frames for each of the analyzed nuclei are included in Fig1.
489 Statistical analysis was applied independently to each brain region (independent t-test for parametric data or Mann-
490 Whitney test for non-parametric data, see Results). *P ≤ 0.05; **P ≤ 0.01; (*) P ≤ 0.06.

491 **C. Effect of testosterone withdrawal in PRL-derived signalling in the male mouse brain**

492 Finally, we explored the putative role of testosterone in the regulation of PRL-derived signalling in
493 the male brain. To do so, we compared pSTAT5-ir patterns between intact, PRL-supplemented males
494 (Male+PRL; Fig 2, second column from left) and a sample of castrated, PRL-supplemented males
495 (Castrated+PRL; Fig 2, third column). In qualitative terms, castrated and intact male mice displayed
496 similar patterns of pSTAT5-ir, but castrated males showed apparently lower levels of pSTAT5-ir.
497 Quantitative analysis of the labelling in both groups (using the same methodology and exploring the
498 same subset of nuclei analysed in the previous section) allowed comparing pSTAT5-ir between
499 gonadally intact and castrated males by means of independent t-tests for the Arc and AVPe and non-
500 parametric Mann-Whitney tests for the rest of the analysed regions. The results of this statistical
501 comparison reveal a significant effect of testosterone withdrawal in diminishing the levels of
502 pSTAT5-ir in the Pa (U= 33.00; p=0.004), in both the ventrolateral and central/dorsomedial VMH (U=
503 30.00, p=0.015 for both frames) and in the SFi (U= 26.00, p=0. 05). In the remaining analysed nuclei,
504 the density of pSTAT5-ir cells was similar in Male+PRL and Castrated+PRL animals, as no statistical
505 differences were found (p>0.1) (Fig 4).



506

507 **Figure 4. Effect of testosterone withdrawal in pSTAT5 immunoreactivity of the male mouse brain**

508 Assessment of pSTAT5-ir density (pSTAT5-positive cell nuclei/mm²) in gonadally-intact male mice and castrated male
509 mice within the major brain regions showing pSTAT5 expression in the male mouse brain. Bar histograms show mean
510 interhemispheric pSTAT5-ir density ± SEM in intact, PRL-supplemented males (Males+PRL; n=6; black) and

511 castrated, PRL-supplemented males (Castrated+PRL; n=6; white). Counting frames for each of the analyzed nuclei
512 are enclosed in Fig 2. Statistical analysis was applied independently to each brain region (independent t-test for
513 parametric data or Mann-Whitney test for non-parametric data). *P ≤ 0.05; **P ≤ 0.01.

514 **DISCUSSION**

515 The present study examined, for the first time, the responsiveness of the brain of males to PRL.
516 Specifically, we analysed the distribution and steroid regulation of PRL-derived signalling in the male
517 mouse brain, by means of the immunohistochemical detection of pSTAT5. We also compared the
518 pattern of pSTAT5 immunoreactivity in the brain of males and females, aiming at assessing its
519 possible sexual dimorphism. In this section, we will first analyse the distribution of pSTAT5 in the
520 brain of male mice, with or without exogenous PRL administration, and compare it to the pattern of
521 expression of the PRLR, as shown in in situ hybridisation material provided by the Allen Brain
522 Institute (see Fig 1 and Fig S2). Then, we will comment on the observed sexually dimorphic
523 differences in central sensitivity to PRL. Next, we will discuss the role of testosterone as a putative
524 regulator of central PRL signalling and on the mutual regulatory relationship of PRL and
525 testosterone. We will conclude by reviewing how our findings contribute to elucidate the
526 neuroanatomical substrate of some putative actions of PRL in the brain of males.

527 **Prolactin signalling in the male mouse brain, PRL supplementation and PRL receptor expression**

528 Although the actions of PRL in the female mouse brain have received considerable attention due to
529 the role of PRL in female reproduction and motherhood(34), this work is, to our knowledge, the first
530 descriptive analysis of the distribution of PRL-derived signalling in the male mouse brain. In PRL-
531 supplemented male mice (Male+PRL group), brain regions positively labelled for pSTAT5 were
532 located mainly in the hypothalamus (the AVPe and VMPO, MPA, Pe, Pa, Arc, DM and VMH) and, to a
533 lesser extent, in the telencephalon (SFi in the septum and MePD in the amygdala), with no additional
534 pSTAT5-ir detected in any of the remaining brain divisions (Fig 1).

535 Importantly, this pattern of pSTAT5-ir was highly dependent on systemic supplementation with a
536 high dose of exogenous PRL, whereas non-supplemented controls (Male Control group) were
537 virtually devoid of pSTAT5-ir, except for some faintly labelled cells in the ventral Arc (Fig 2T) and in
538 the anterior portion of the periventricular hypothalamus (not shown). These results suggest, in the
539 first place, that the patterns of pSTAT5-ir we report in this work are specifically produced by PRL,
540 whereas other endocrine agents signalling through the Jak/STAT pathway, such as growth
541 hormone(35), do not contribute substantially to STAT5 phosphorylation in the studied cases. This is
542 further supported by the fact that pSTAT5 appears only in centres that show expression of PRLR in
543 the brain of male mice (Fig 1, Fig S2), whereas the distribution of growth hormone receptor does
544 not fit the pattern of pSTAT5-ir(35,36). In addition, the reduced pSTAT5-ir found in males not
545 supplemented with exogenous PRL indicates very low levels of PRL signalling in basal conditions,
546 what fits the circulating levels reported for the hormone for males(10,37). In fact, the dorsal aspect
547 of the Arc (presumed location of the TIDA neurons as revealed by tyrosine hydroxylase
548 immunohistochemistry, 38), displays scarce pSTAT5-ir in Male Control mice (Fig 2T).

549 Altogether, the male mouse brain shows little responsiveness to PRL under basal conditions, but this
550 responsiveness would increase significantly when higher levels of the hormone reached the brain.
551 Hence, PRL would influence the brain of males especially under some physiological conditions in
552 which there is substantially increased hypophyseal or extra-hypophyseal PRL release, for instance
553 during the dark period of the day (37), after mating(39) or as part of the stress response (40).

554 **Sexually dimorphic PRL-derived signalling in the mouse brain**

555 In comparison to males, female mice displayed a more extensive distribution of pSTAT5-ir, mainly in
556 the hypothalamus and basal telencephalon, but also in different nuclei of the thalamus, midbrain
557 and brainstem. Patterns of pSTAT5-ir in males were always a completely overlapping fraction of
558 those of female mice, with no single brain structure labelled in males but not in females.
559 Furthermore, a number of nuclei with pSTAT5 expression in both sexes displayed significantly higher

560 pSTAT5-ir density in females than in males (the AVPe/VMPO, Arc, DM and MePD), but none of the
561 analysed nuclei showed higher levels of pSTAT5-ir in males (Fig 3). Altogether, our study reveals a
562 clear, female-biased sexual dimorphism in PRL-derived signalling in the mouse brain upon exogenous
563 PRL administration. In fact, this sexually dimorphic responsiveness to PRL would be extensive to
564 specific functional systems of the brain, for instance to the Sociosexual Behavioural Network (SBN),
565 the core system for the integration of social and reproductive behaviour (41). In female mice,
566 virtually all nodes of the SBN display apparent PRL responsiveness (4), whereas males lack pSTAT5-ir
567 or display marginal levels thereof (MePD) in all of them (the lateral septum, BST, medial amygdala or
568 PAG, see Figs 1 and 2) except for the VMH, even after exogenous PRL administration and even if they
569 express the PRL receptor (Fig 1, Fig S2). This suggests that, in these areas, either PRL responsiveness
570 is regulated through different receptor activation pathways in males and females, or PRL signalling is
571 downregulated, leading to a lower implication of PRL in the regulation of sociosexual behaviours in
572 males. However, this does not exclude a role in some male-specific behaviours, as we will further
573 discuss below.

574

575 **Prolactin and Testosterone: Mutual Neuroendocrine Regulation**

576 One of the main outcomes of the present work is the identification of a putative regulatory role of
577 testosterone in PRL signalling in the male mouse brain. Orchidectomy and consequent testosterone
578 withdrawal led to a significant decrease in pSTAT5-ir density in the SFi, the Pa and both the
579 central/dorsomedial and lateral VMH (Fig 4). This indicates a positive regulatory role of testosterone
580 on PRL signalling in those brain regions. The actual (direct or indirect) mechanism of this process is
581 not clear. Even though testosterone has documented inhibitory effects on hypophyseal PRL release
582 in male rats(29) and mice(30), the effect of castration on pSTAT5-ir cannot be attributed to
583 differences in circulating PRL, since intact and castrated males were supplemented with equivalent
584 levels of exogenous PRL. In addition, this effect is not likely explained by a facilitated access of

585 systemic PRL to the brain, as our results reveal that the positive effect of castration on pSTAT5-ir is
586 not generalised but rather specific and restricted to discrete brain sites. Therefore, our results
587 suggest that testosterone might upregulate PRLR expression or perhaps signalling downstream the
588 PRLR in specific neural populations. Further research is required to elucidate the specific action of
589 testosterone on PRL signalling in male mice.

590 An additional question of interest is the identity of the actual biological modulator of PRL signalling
591 we report here. Testosterone can be locally metabolized into two different neuroactive steroids: it
592 can be reduced to dihydrotestosterone (42), which binds to androgen receptors, or aromatised to
593 estradiol (43) that then binds to oestrogen receptors. Our results make it very unlikely that this
594 regulation occurs through the aromatisation of testosterone to estradiol. Although all the nuclei
595 where PRL signalling is affected by castration express at least one form of oestrogen receptor(44),
596 none of them displays aromatase activity in the male mouse brain(45). Conversely, all of the nuclei
597 where PRL-derived signalling decreased with castration express androgen receptors, with the Pa and
598 VMH showing very high levels(45). Hence, evidence suggests that the effect of testosterone on PRL
599 signalling that we report here is likely mediated by androgen rather than oestrogen receptors.

600 In addition to the reported regulation of testosterone on central PRL signalling, our data, together
601 with published evidence, suggest a reciprocal regulatory action of PRL on the hypothalamus-
602 pituitary-gonadal (HPG) axis and ultimately on testosterone release. According to the literature, PRL
603 is thought to upregulate the HPG axis and testosterone secretion in males, since PRL infusions
604 stimulate the increase in serum testosterone in male rats, whereas immunological disruption of
605 endogenous prolactin eliminates this effect (14,46). This regulatory action of PRL on the HPG axis
606 likely occurs through the kisspeptin system, which regulates gonadotropin-releasing hormone
607 (GnRH) expressing neurons in the brain(47) and thereby activates the HPG axis. Therefore, the
608 expression of PRL receptors by kisspeptin neurons(48) allows for a direct role of PRL on HPG axis
609 control and testosterone secretion. In this context, our results provide support for the hypothesis

610 that the brain kisspeptin system is subject to PRL regulation in male mice, too, since our sample of
611 male mice displayed substantial pSTAT5-ir in the hypothalamic nuclei containing kisspeptin-positive
612 neurons(49), the AVPe and the Arc (Figs 2j and 2r). Nevertheless, this possibility requires
613 experimental proof by assessing the coexpression of kisspeptin and pSTAT5 immunoreactivity in the
614 aforementioned nuclei.

615 **Prolactin signalling in the male brain: functional implications**

616 Our findings confirm that the male mouse brain is indeed responsive to PRL, implying that PRL likely
617 exerts certain actions in the male mouse brain, provided sufficient levels of the hormone. The nature
618 of these actions is mostly unknown, but the multiple functional studies on central PRL in female mice
619 provide a good comparative framework to contextualize our findings.

620 In this respect, the most prominent (but poorly understood) function of PRL in males is the
621 regulation of sexual behaviour (3,16). It has been shown that central action of PRL after mating
622 suppresses sexual behaviour as part of the satiatory mechanisms operating after ejaculation (17,22).
623 Similar inhibitory action of PRL onto sexual behaviour occurs under chronic hyperprolactinaemia(50–
624 52). Prolactin has been proposed to do so by modulating the function of the major dopaminergic
625 circuits in the brain, closely involved in the motor component of male sexual function (17, 53). In
626 support of this, acute elevations of circulating PRL after copulation lead to significant decreases in
627 dopaminergic activity in the target sites of the major dopaminergic pathways (17), including the
628 striatum (23,54) and the MPA region (55). Several studies have shown that, indeed, PRL is capable of
629 modulating the dopaminergic activity of these pathways at several levels (54–58). However,
630 according to our findings, none of the nuclei originating these dopaminergic pathways (17) display
631 PRL-derived signalling neither in female nor in male mice, even after injection of exogenous PRL (Figs
632 1 and 2). Furthermore, in situ hybridisation in the mouse brain does not show any expression of
633 PRLR mRNA within these pathways, neither in their sites of origin (substantia nigra or ventral
634 tegmental area), nor in their projection sites (caudatus putamen or nucleus accumbens) (Fig S2),

635 although certain studies did find evidence of the receptor expression in the rat (59,60). Hence, this
636 suggests that the proposed action of PRL over these dopaminergic systems to regulate male sexual
637 behaviour should rather be indirect. In this vein, our study evidences a testosterone-dependent
638 action of PRL on two nuclei of the male brain that have also been involved in male sexual behaviour
639 regulation: the VMH and the Pa (Figs 2 and 4). The VMH is sexually dimorphic (61), known to
640 promote the expression of copulatory behaviour in females (62) and to inhibit mounting in male rats
641 (63), whereas the Pa contains magno- and parvocellular oxytocin (OXT) neurons involved in the
642 peripheral and central regulation of male sexual behaviour, respectively (64–67).

643 The interaction between OXT and PRL at the level of the Pa can also have a role in the regulation of
644 parental behaviours. There is solid evidence indicating that PRL (68) and OXT are fundamental in the
645 control of maternal behaviour (69,70) and it is likely that they have an equivalent role in males. In
646 this line, PRL facilitates pup-sensitisation in male rats (71) in a similar fashion as in females (68) and
647 has further roles in other experimental models for paternal behaviour (72). Tachikawa and
648 collaborators (73) showed that paternal behaviour can be induced in male mice (which are usually
649 infanticidal) by postcopulatory cohabitation with a maternal female, a process apparently
650 dependent on OXT release in the nucleus accumbens (Acb) (74,75). In turn, Dolen and collaborators
651 (76) demonstrated that the OXT innervation of the Acb originates in the Pa (see also(77), which,
652 according to our findings, is responsive to PRL in males (Fig 2M). Therefore, PRL might participate in
653 the regulation of parental behaviour in male mice, too, by acting on Pa OXT neurons. This hypothesis
654 requires further study.

655 Our results indicate a modulatory role of PRL onto the Arc and Pe nuclei of males (2R-T), both of
656 which contain the dopaminergic neuron populations responsible for the inhibitory feedback control
657 of hypophyseal PRL release(78): the tuberoinfundibular (TIDA) and tuberohypophyseal (THDA)
658 neurons in the dorsomedial and rostral Arc(79), and the periventricular hypophyseal neurons (PHDA)
659 in the Pe(80). In fact, in female rats PRL feedback signal in these dopaminergic populations is

660 conveyed through the Jak/STAT pathway, specifically through STAT5b (81). Therefore, our results in
661 the Arc and Pe likely reflect the (expected) occurrence of the same feedback mechanism in male
662 mice.

663 Prolactin also plays an important role in the modulation of anxiety and stress. Concerning anxiety,
664 studies in female rats indicate that PRL has anxiolytic properties when administered i.c.v. or i.v.,
665 whereas chronic i.c.v. antisense blocking of the PRLR expression results in increased anxiety(82). In
666 contrast to females, PRL has a weak anxiolytic effect in males(82). This intersexual difference might
667 relate to our findings of a sexually dimorphic responsiveness to PRL in the central structures directly
668 involved in the regulation of anxiety and emotionality, the Ce and the anterodorsal BST (83). In these
669 nuclei, males are devoid of pSTAT5-ir (Fig 1) although they show PRLR mRNA transcript (Fig 1 and fig
670 S2), whereas females show quite abundant immunostaining, especially during pregnancy and
671 lactation (4).

672 Besides being a central agent in acute stress response (11), PRL also has an important role in stress
673 modulation. Thus, central inhibition of the expression of PRLR by i.c.v. antisense blocking results in
674 an enhanced activation of HPA axis in male and female rats (82), demonstrating a downregulation of
675 the stress response by PRL. The presence of pSTAT5-ir in the Pa of males (in levels equivalent to
676 females, see Figs 2m, 2n and 3) suggests that this modulatory effect of PRL on the reactivity of the
677 HPA axis would probably take place through PRL action on corticotropin-releasing factor (CRF) or
678 vasopressin (AVP) cells (84). In fact, PRL is a well-documented regulator of the function of magno-
679 and parvocellular vasopressinergic neurosecretory cells at the level of the hypothalamic Pa (85–87).
680 Although effects of PRL on these parvocellular AVP cells has been proven to be mediated by
681 ERK/MAPK pathway (87), our data in males and females suggest that the Jak/STAT5 signalling
682 cascade can also be involved. This would imply a non-dimorphic regulatory role of PRL on the stress
683 response (in addition to its dimorphic role on anxiety), which requires further research.

684 **Conclusions**

This work characterizes the distribution of PRL responsiveness in the male mouse brain and its dependence on circulating PRL levels, sexual dimorphism and gonadal steroid regulation. Male mice display specific patterns of PRL-derived signalling only in the presence of high levels of circulating PRL. These patterns comprise mainly hypothalamic nuclei and some telencephalic sites, and are also more limited in extension and density than the equivalent patterns in female mice, evidencing a clear sexual dimorphism in favour of females. Furthermore, PRL-derived signalling in the male brain is regionally dependent on testosterone input, indicating a reciprocal regulation between both hormones. This work confirms that PRL exerts central actions in males, suggesting a possible role of the "maternal hormone" in the regulation of male sexual behaviour, PRL release feedback control or in the regulation of stress response, among others.

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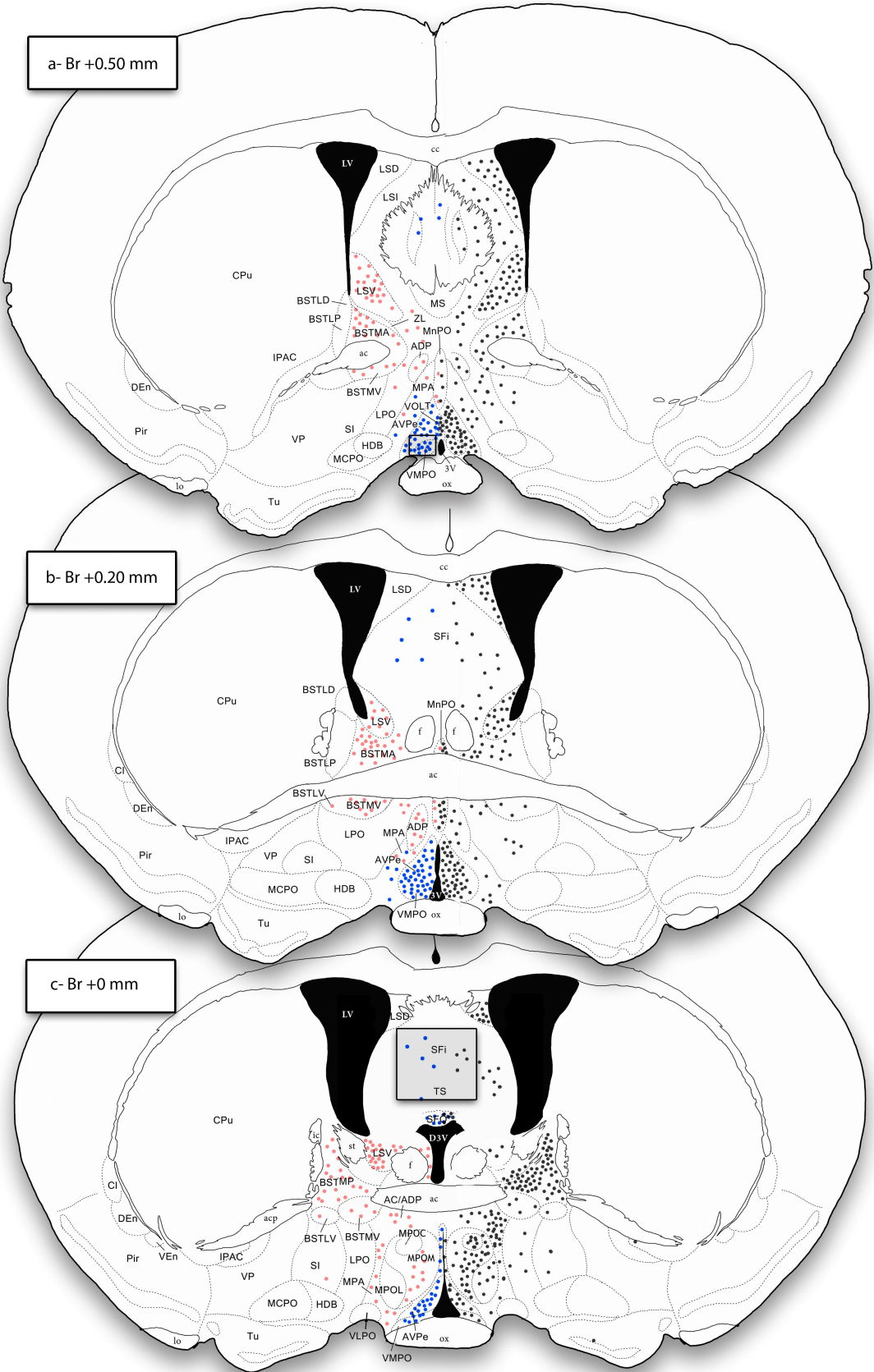
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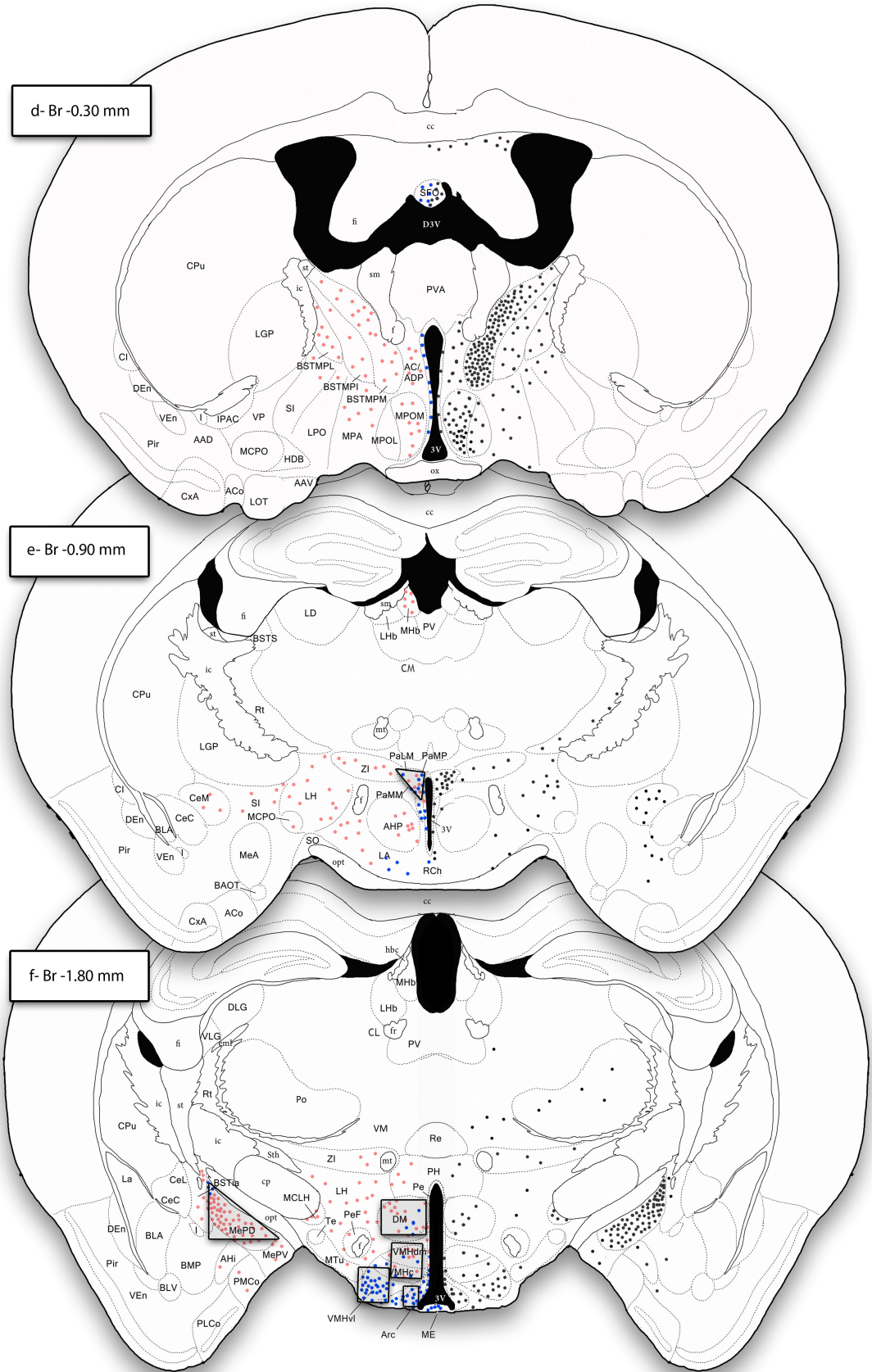
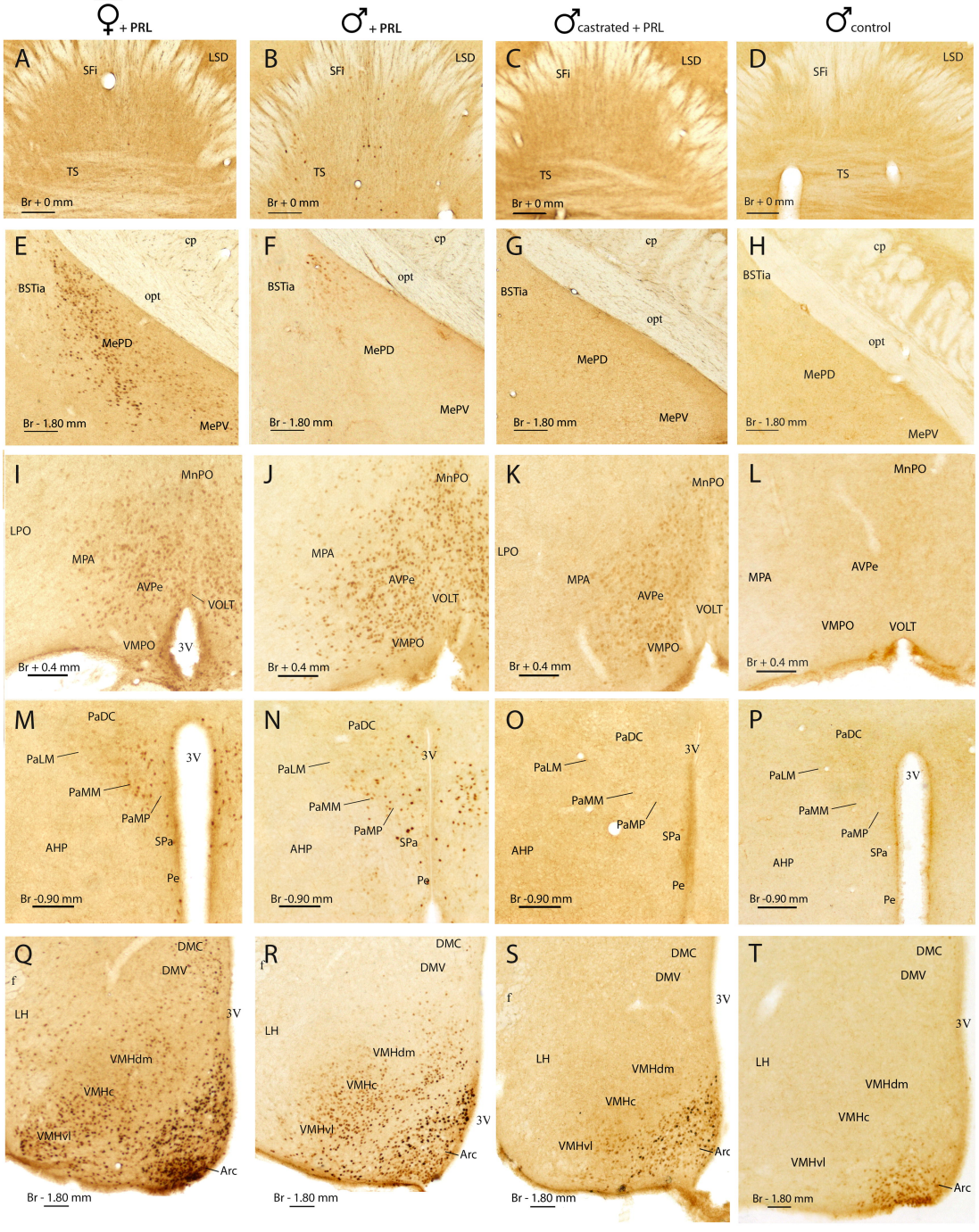
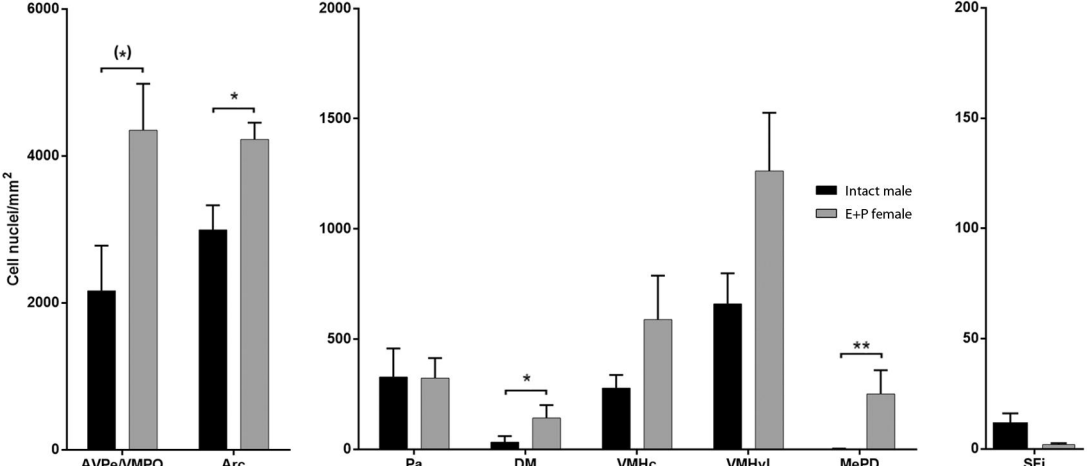
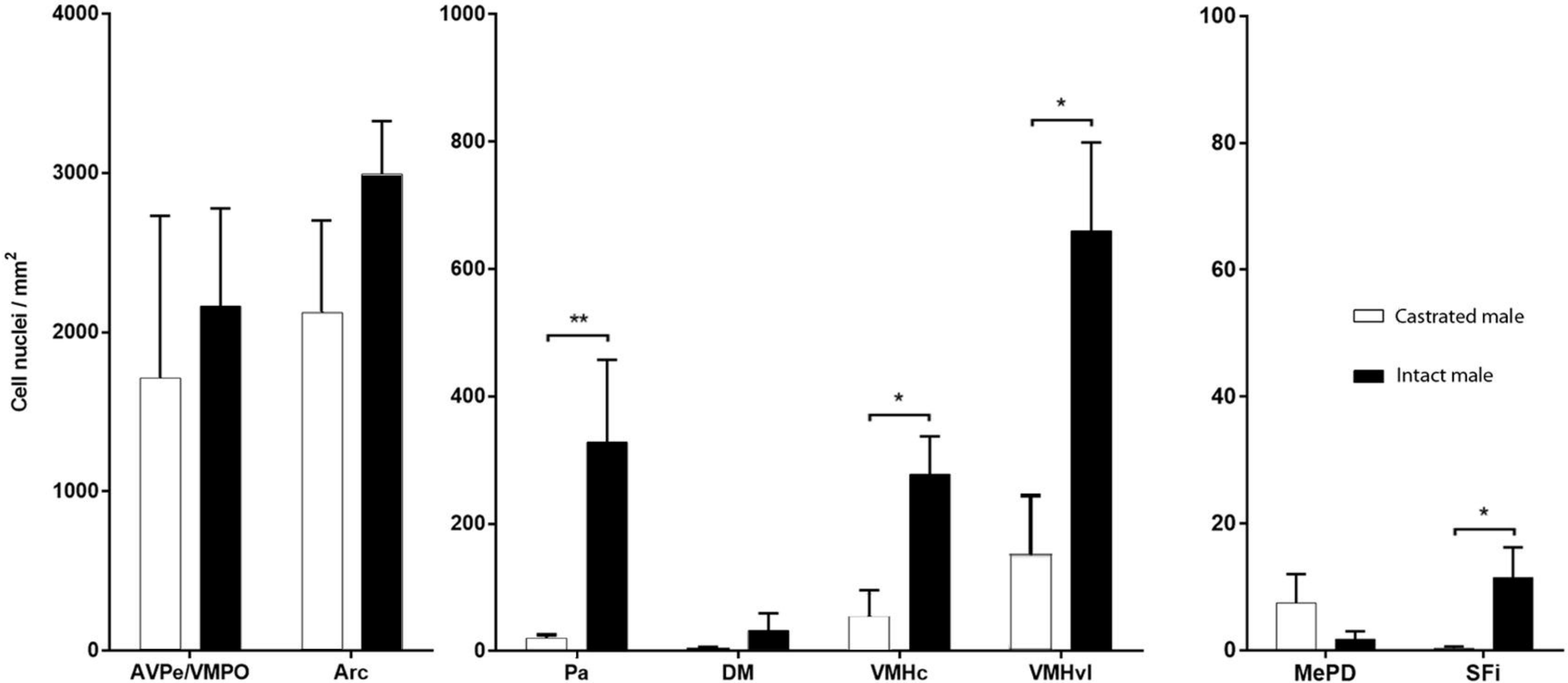
AC/ADP
ac
DEn
acp
BSTLV
BSTMV
MPOC
Pir
VEn
IPAC
MPOM
SI
LPO
VP
MPA
MPOL
MCPO
HDB
lo
Tu
VLPO
AVPe
ox
VMPO


d- Br -0.30 mm
cc
SFO
fi
D3V
CPu
st
sm
ic
PVA
LGP
f
Cl
BSTMPL
AC/
ADP
DEn
BSTMPI
BSTMPM
VEn
I
IPAC
VP
SI
MPOM
Pir
AAD
LPO
MPA
MPOL
3V
MCPO
HDB
ox
AAV
CxA
ACo
LOT
e- Br -0.90 mm
cc
fi
LD
sm
st
BSTS
LHb
MHb
PV
ic
CM
CPu
Rt
mt
LGP
PaLM
PaMP
ZI
Cl
CeM
SI
LH
f
PaMM
DEn
CeC
MCPO
AHP
3V
BLA
Pir
VEn
I
MeA
SO
opt
LA
RCh
BAOT
ACo
CxA
cc
f- Br -1.80 mm
hbc
MHb
DLG
LHb
CL
fr
fi
VLG
cml
PV
Rt
Po
ic
st
CPu
VM
Re
ic
Sth
ZI
mt
PH
La
CeL
BSTia
cp
Pe
CeC
MCLH
LH
opt
PeF
DM
DEn
BLA
I
MePD
Te
f
VMHdm
Pir
BMP
AHi
MePV
MTu
VMHc
PMCo
BLV
VEn
3V
VMHvl
PLCo
Arc
ME


♀ + PRL
♂ + PRL
♂ castrated + PRL
♂ control
A
SFi
LSD
TS
Br + 0 mm
B
SFi
LSD
TS
Br + 0 mm
C
SFi
LSD
TS
Br + 0 mm
D
SFi
LSD
TS
Br + 0 mm
E
cp
BSTia
opt
MePD
MePV
Br - 1.80 mm
F
cp
opt
BSTia
MePD
MePV
Br - 1.80 mm
G
cp
BSTia
opt
MePD
MePV
Br - 1.80 mm
H
cp
BSTia
opt
MePD
MePV
Br - 1.80 mm
I
MnPO
LPO
MPA
AVPe
VOLT
VMPO
3V
Br + 0.4 mm
J
MnPO
MPA
AVPe
VOLT
VMPO
Br + 0.4 mm
K
MnPO
LPO
MPA
AVPe
VOLT
VMPO
Br + 0.4 mm
L
MnPO
MPA
AVPe
VMPO
VOLT
Br + 0.4 mm
M
PaDC
PaLM
3V
PaMM
PaMP
AHP
SPa
Pe
Br -0.90 mm
N
PaDC
3V
PaLM
PaMM
PaMP
AHP
SPa
Pe
Br -0.90 mm
O
PaDC
3V
PaLM
PaMM
PaMP
SPa
AHP
Pe
Br -0.90 mm
P
PaDC
PaLM
3V
PaMM
PaMP
SPa
AHP
Pe
Br -0.90 mm
Q
DMC
DMV
f
LH
3V
VMHdm
VMHc
VMHvl
Arc
Br - 1.80 mm
R
DMC
DMV
f
LH
VMHdm
VMHc
3V
VMHvl
Arc
Br - 1.80 mm
S
DMC
DMV
f
3V
LH
VMHdm
VMHc
VMHvl
Arc
Br - 1.80 mm
T
DMC
DMV
3V
LH
VMHdm
VMHc
VMHvl
Arc
Br - 1.80 mm


Cell nuclei/mm2
6000
4000
2000
0
(*)
*
AVPe/VMPO
Arc
2000
1500
1000
500
0
*
**
Intact male
E+P female
Pa
DM
VMHc
VMHvl
MePD
200
150
100
50
0
SFi


Cell nuclei / mm2
4000
3000
2000
1000
0
AVPe/VMPO
Arc
1000
800
600
400
200
0
**
*
*
Pa
DM
VMHc
VMHvl
100
80
60
40
20
0
*
MePD
SFi
Castrated male
Intact male